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A novel *in vivo* model using immunotoxin in the absence of p-glycoprotein to achieve ultra selective depletion of target cells: applications in trogocytosis and beyond

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Abstract

A commonly employed method to determine the function of a particular cell population and to assess its contribution to the overall system*in vivo* is to selectively deplete that population and observe the effects. Using monoclonal antibodies to deliver toxins to target cells can achieve this with a high degree of efficiency. Here, we describe an *in vivo* model combining the use of immunotoxins and multidrug resistant (MDR) gene deficient mice so that only MDR deficient cells expressing the target molecule would be depleted while target molecule expressing, but MDR sufficient, cells are spared.This allows targeted depletion at a higher degree of specificity than has been previously achieved. We have applied this technique to study trogocytosis, theintercellular transfer of cell surface molecules, but this principle could also be adapted using technology already available for use in other fields of study.

Key words: Immunotoxin, cell depletion, trogocytosis, molecular transfer, antigen presentation

Abbreviations: MDR, Multidrug Resistant

1. Introduction

Currently, one of the most effective ways to remove a specific cell population for *in vivo*and *in vitro* studies is by using depleting monoclonal antibodies; however, this can also cause inflammation through antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity.As an alternative, transgenic mice expressing the diphtheria toxin receptor under a relevant promotermay be utilised, in which the specific cell populationisdepleted after diphtheria toxin administration (1, 2).Cre or CreERT2 / loxP systems are also commonly used.

We describe a new*in vivo*method of depleting specific cell populations with an extra degree of flexibility by usingantibody based immunotoxin technology in the context of multidrug resistant (MDR) gene deficiency.Our intention is to use this new technique to study intercellular molecular transfer. However, this principle can be modified, using existing technology, to achieve depletion of highly specific populations of cells *in vivo* for other types of studies.

Cell surface molecules play a major role in the functioning of a cell, controlling cell function through signalling and allowing cell-to-cell communication. Therefore, the discovery of trogocytosis (3, 4) – the transfer between cells of portions of cell membrane including surface molecules - unlocks the possibility of many and varied physiological consequences, which has implications for all fields of biomedical research. Molecules shown to be transferred between cells include MHC class I and II, CD3, co-stimulatory molecules, endothelial cell molecules, natural killer (NK) receptors, chemokine receptors, and GPI-anchored proteins (5,6,7); subsequent work has suggested that it may be through a Fcγ-receptor mediated process (8).

Consequences of the transfer of cell surface molecules have been studied extensively *in vitro*. In terms of MHC class II molecules, it has been shown that transfer can result in either amplification or downregulation of the immune response (5,6).The differing outcomes are probably caused by differing environmental conditions at different stages of the immune response. The outcome could also be altered by the regulation of transfer. The possible widespread consequences of the transfer of cell surface molecules suggests that this process will be tightly regulated, and indeed it is known that transfer is limited to cells that have close contact, that not all cell

surface molecules can be transferred, and that the duration of expression of the molecule on the recipient cell can be limited.

Such a complex array of factors isdifficult to recreate *in vitro*. Therefore, to gain insight into the real physiological relevance of intercellular molecule transfer, *in vivo* studies are necessary. It has been shown that adoptively transferred memory, but not naive, $CD8^+$ T cells proliferate in response to antigen presenting cells that had acquired peptide/MHC class I molecules from parenchymal cells (9). NK cells that had acquired Rae-1 from tumour cells were killed *in vivo* by fratricide upon injection into naive mice (10). In transplantation, it has been shown *in vivo* that recipient T cells acquire peptide/MHC class I from graft parenchyma and present it in secondary lymphoid tissues to $CD8^+$ T cells, while also presenting processed donor peptide on recipient MHC class II for $CD4^+$ T cells (11). This has shown a functional role for MHC transfer *in vivo* after transplantation;however, the complex models used did not allow its contribution to the normal alloresponse to be assessed. To study this, we have used a mouse organ transplantation model and adopted an antibody-directed immunotoxin approach to deplete recipient cells that have acquired donor MHC class II.

An anti-donor MHC class II antibody was used to direct a “toxin” towards cells that express donor class II MHC molecules. If the donor (but not recipient) cells are resistant to the toxin, then only recipient cells that have acquired donor class II MHC molecules will be lysed (Fig 1), thus achieving specificity that is not possible using depleting antibody alone.

The widely expressed multidrug resistant (MDR) gene (12) encodes a membrane-spanning transporter molecule (P-glycoprotein (Pgp)), that in normal cells expels a variety of drugs out of the cell. This confers a degree of resistance to moderate quantities of cytotoxic drugs such as gelonin, a ribosome inactivating protein. Conversely, $MDR^{-/-}$ cells are exquisitely sensitive to killing by toxin due to the inability to remove toxins from the intracellular compartment. In addition, lysosomal accumulation of immunotoxin complexs has been identified as a mechanism of toxin sensitivity in $MDR^{-/-}$ cells. As a result, $MDR^{-/-}$ mice are extremely sensitive to even small doses of the same drugs (12,13). MDR^+ cells have been shown to be resistant to a

gelonincontaining immunotoxin while $MDR^{-/-}$ cells are killed (14). A number of immunotoxins are now in clinical use and have been shown to be safe and effective(15).

Here, we transplanted MHC mismatched wild type donor organs into $MDR^{-/-}$ recipients. We show that upon administration of low doses of an anti-donor MHC class II immunotoxin, recipient cells that have acquired donor MHC molecules are depleted.

2. Materials and Method

2.1 Anti I-A^k-gelonin immunotoxin preparation

A mouse anti-mouse MHC class II I-$A^{k,r,f,s}$ IgG2a monoclonal antibody (Clone 10-3.6.2, BioXCell, West Lebanon, NH, US) was digested into F(ab')$_2$ and Fc fragments using an F(ab')$_2$ preparation kit (Pierce (Thermo Fisher Scientific), Cramlington, UK) according to manufacturer's instructions. Recombinant gelonin (rGel) was synthesised as previously described (15). Conjugation of F(ab')$_2$ fragments and rGel was performed as previously described (14). Briefly, 3-(2-Pyridyldithio) propionic acid N-hydroxysuccinimide ester (SPDP, Sigma, Gillingham, UK) was dissolved 3mg/ml in dimethylformamide and 30µl was immediately added to 1ml of F(ab')$_2$ fragments at a concentration of 5mg/ml. The reaction mixture was vortexed regularly over 30 minutes at room temperature. Unreacted SPDP was removed by passage through a PD-10 column (GE Healthcare, Little Chalfont, UK). 20mM dithiothreitol (Sigma) in PBS was added to 5mg rGel at a volume of 0.1x rGel volume. The reaction mixture was vortexed regularly over 30 minutes at room temperature. Unreacted dithiothreitol was removed by passage through a PD-10 column. Both modified F(ab')$_2$ fragments and rGel were eluted from the PD-10 columns in 0.1M sodium phosphate, 0.15M NaCl, 10mM EDTA, pH 7.5, which had been degassed in a vacuum oven prior to use. After elution, the modified F(ab')$_2$ fragments and rGel were mixed together (resulting in a 4-fold molar excess of rGel over F(ab')$_2$ fragments), concentrated using a Vivaspin 6 centrifugal concentrator (10kDa MWCO, Sartorius Stedim UK Ltd, Epsom, UK), centrifuged at 2000 rcf for 45 minutes at 4^0C, and then incubated for 20 hours at 4^0C. To stop the reaction, iodoacetamide was added to the solution for 1 hour at room temperature, to give a final concentration of 2mM.

Unconjugated gelonin was removed from the reaction mixture by passage through a HiTrapr Protein A FF column (SLS, Nottingham, UK), using the equilibration buffer 50 mM sodium phosphate, 0.15M NaCl, pH 7.5 and the elution buffer 0.1M acetic acid, 0.15M NaCl according to manufacturer's instructions. Unconjugated F(ab')$_2$ fragments were removed by passage through a HiTrap Blue HP column (SLS), using the equilibration buffer 20mM sodium phosphate, 0.6M NaCl and the elution buffer 20mM sodium phosphate, 2M NaCl according to manufacturer's instructions. Yield of Anti I-A^k-gelonin was determined by absorbance at 280nm.

2.2 SDS PAGE

Antibody, F(ab')$_2$ fragments, and conjugated Anti I-A^k-gelonin were run alongside a SeeBlue Plus2 Pre-stained standard (Life Technologies), on a NuPAGENovex 4-12% bis-tris gel (Life Technologies, Paisley, UK) at 180 volts for 40 minutes. The gel was then rinsed and incubated with Simply Blue Safe Stain (Life Technologies) for 2 hours with rocking.

2.3 Animals

All animals were between the age of 8 and 12 weeks and used in accordance with the Animals (Scientific Procedures) Act 1986. Female C57BL/6 x CBA F_1 (H-2$^{b/k}$), FVB (H-2^q), and CBA.Ca (H-2^k) mice were purchased from Harlan Limited (Bicester, UK). FVB background MDR1a/b-Bcrp constitutive knockout mice (abbreviated as MDR$^{-/-}$), deficient in the P-glycoprotein membrane-spanning transporter molecule, were purchased from Taconic Europe (Laven, Denmark) and then bred in-house. C57BL/6 mice expressing the enhanced yellow fluorescent protein (EYFP) transgene on all cells (rosa26 promoter knockin) and C57BL/6 EYFP x CBA F1 mice were bred in-house. All mice were kept under specific pathogen-free conditions.

2.4 Binding assay

To determine binding, antibody, F(ab')$_2$ fragments or conjugated Anti I-A^k-gelonin were added to CBA cells (which express I-A^k). After a 30 minute incubation, unbound material was washed off and one of 2 antibodies were added – an anti-I-A^k-FITC (clone 10-3.6, Biolegend, London, UK), which is the same clone as the antibody used for conjugation, and an anti-I-A^k-PE (clone 11-5.2, Biolegend), which is an irrelevant clone and whose binding should not be inhibited by the previous incubation. Cells were acquired using a FACScalibur flow cytometer (BD, Oxford, UK) and analyzed using Cellquest software version V3.3 (BD).

2.5 *In vivo* killing assay – splenocyte injection

The effectiveness of the Anti I-A^k-gelonin in killing cells *in vivo* was tested. Single cell suspensions were obtained from spleens harvested from BL/6 EYFP x CBA F1 mice. Red blood cells were lysed and the

splenocytes injected intravenously into recipient $MDR^{-/-}$ mice (one spleen per recipient). Anti I-A^k-gelonin was then injected intravenously into these mice. Spleens of recipient mice were harvested 20 hours later and flow cytometry performed, using an anti-I-E^k-PE antibody (clone 14-4-4S, eBioscience, Hatfield, UK), which recognises the other MHC class II molecule on target cells so that binding of Anti I-A^k-geloninto the target cells would not hinder the detection of MHC class II positive cells. Cells were acquired as before.

2.6 5-bromo-2'-deoxyuridine administration

To label dividing cells in donor animals, 5 week old BL/6 x CBA F1 mice were housed in cages with drinking water containing 5-bromo-2'-deoxyuridine (BrdU) dissolved in tap water at a concentration of 1mg/ml. Dark coloured water bottles were used to prevent BrdU being deactivated by exposure to light, and the water was changed twice per week. After 21 days of BrdU treatment mice were used as organ donors.

2.7 Organ transplantation

Mouse renal and heart transplantation were performed as described previously (17, 18). I-A^k-gelonin, when administered, was injected intravenously in a single dose following transplantation.

2.8 *In vivo* killing assay – organ transplantation

One day after transplantation of hearts or kidneys from BrdU-treated BL/6 x CBA F1 mice, spleens and transplanted organs were harvested and frozen in optimal cutting temperature compound (OCT, VWR, Lutterworth, UK). 5µm-thick section were cut and left to air dry overnight. Sections were fixed in ice-cold acetone for 5 minutes and air dried. Endogenous peroxidase activity within the tissue was quenched with 0.3% H_2O_2 in PBS for 10 mins, and the sections were rinsed in PBS, before endogenous biotin was blocked with an avidin/biotin blocking kit (Vector Laboratories, Burlingame, USA). Biotinylated anti-I-A^b (clone AF6-120.1, BD) was diluted 1 in 100 in PBS and added to sections for 1 hour, followed after washing with PBS by streptavidin-horseradish peroxidise (BD) for 30 minutes. Colour was developed using a NovaRED peroxidase substrate kit (Vector Laboratories). Sections were washed in PBS and any unbound biotin blocked with the avidin/biotin blocking kit. BrdU staining was then performed using the BrdU*in-situ* detection kit (BD)

according to manufacturer's instructions. Slides were placed in preheated Retrievagen A solution and returned to a water bath set at 89^0C for 20 minutes. Slides were then removed from the water bath but kept in the Retrievagen A solution for 20 minutes to allow them to return slowly to room temperature. After washing in PBS, anti-BrdU antibody diluted 1:10 was added to sections for 1 hour, followed after washing with PBS by streptavidin-PE (BD), diluted 1:100 in PBS for 30 minutes in the dark. After a final wash in PBS sections were mounted in PermaFluor (Fisher Scientific Ltd, Loughborough, UK).

2.9 Statistical analysis

Unpaired two-tailed Student's t-tests were used for all results. Numbers are expressed as means $\pm$ standard error of mean.

3. Results

3.1 Immunotoxin preparation

For any immunotoxin to be effective, the antibody from which it is made must be internalised so that the toxin can be delivered into the cell cytoplasm to exert its effect. The internalisation of the mouse anti-mouse MHC class II I-$A^{k,r,f,s}$ IgG2a monoclonal antibody (clone 10-3.6.2, abbreviated as anti-I-A^{k}) was first tested using I-A^{k} expressing CBA splenocytes as target cells. When FITC labelled anti-I-A^{k} was incubated with target cells *in vitro*, confocal microscopy showed internalisation of this antibody (fig 2a). Although this method does not quantify the amount of antibody that is being internalised, as we aim to use a highly toxic molecule, even a small amount would be sufficient to kill the target cell (19).

Upon intravenous injection of anti-I-A^{k}antibody into I-A^{k} expressing CBA mice, the antibody was found to be depleting. It decreased the amount of I-A^{k} expressing cells in peripheral blood in a dose-dependent manner, by 12.3% at a dose of 0.1 mg, to 32.7% at a dose of 1 mg. To restrict the killing to that mediated by gelonin only, the antibody was digested to remove the Fc portion, leaving the $F(ab')_2$ fragments. A binding assay using CBA target cells showed that $F(ab')_2$ fragments retained the antigen-binding capacity of the whole antibody (fig 3a), as demonstrated by pre-incubation with the $F(ab')_2$ fragments resulting in reduced binding to directly FITC conjugated whole antibody. This reduction was the same as that observed using whole undigested antibody.This experiment was performed twice with various concentrations of antibody and F(ab')2 fragments. In each experiment the percentage of cells bound by the relevant antibody clone decreased in a dose dependent manner and equivalently for antibody and F(ab')2. In another independent experiment, the percentage of positive cells decreased by 22.1, 36.7, 43.5 and 39.6% following pre-incubation with increasing concentrations of antibody, and by 8.4, 36.3, 41.5 and 37.6% following pre-incubation with F(ab')2 fragments at the same concentrations.

Incomplete inhibition after pre-incubation may be due to a number of reasons. 1. Cells analysed by flow cytometry were live cells, in which there is a constant turnover (internalisation and new surface expression) of MHC molecules (as we have demonstrated here infig 2a). It is possible that at least some of the incomplete inhibition was due to new MHC molecules being expressed after the pre-incubated antibody/ $F(ab')_2$

fragmentshad been washed off. 2. Binding of the antibody/ $F(ab')_2$ fragments may be a dynamic equilibrium. Addition of a second antibody may displace at least some of the pre-bound antibody/ $F(ab')_2$ fragments.

The $F(ab')_2$ fragments were conjugated to the toxin gelonin to construct an immunotoxin, abbreviated as I-A^k-gelonin. An SDS page gel of the original antibody, the $F(ab')_2$ fragments, and the prepared conjugate shows the Anti I-A^k-gelonin as a protein slightly larger than the $F(ab')_2$ fragments. After removal of unconjugated gelonin and $F(ab')_2$ fragments from the conjugate preparation only a small band of contaminating $F(ab')_2$ fragments is seen (fig 3b). A binding assay using CBA target cells confirmed that the conjugate retained the antigen-binding capacity of whole antibody (fig 3c). This experiment was performed with 3 concentrations of antibody and conjugate, and the percentage of cells bound by the relevant antibody clone decreased in a dose dependent manner and equivalently for antibody and conjugate. The percentage of positive cells decreased by 27.9, 38.9 and 40.5% following pre-incubation with increasing concentrations of antibody, and by 11.7, 33.1 and 40.1% following pre-incubation with conjugate at the same concentrations. We chose to present the data from just one dose for clarity, and for comparison with fig 3a.

The Anti I-A^k-gelonin immunotoxin has been used in a separate study at high doses (to overcome the expulsion of gelonin from the target cell by wild type cells), in which it was demonstrated to be effective *in vitro* at killing wild type CBA cells expressing target antigen, but not third party cells (20), so we went on to test the immunotoxin *in vivo* in $MDR^{-/-}$ mice to see if it would achieve the high level of specificity required.

3.2 *In vivo* titration of Anti I-A^k-gelonin immunotoxin

C57BL/6 mice expressing the fluorescent gene EYFP ubiquitously were crossed with CBA mice as the EYFP allows tracking of donor cells in recipient mice and crossing these C57BL/6 background mice (H-2^b) with CBA (H-2^k) results in offspring expressing both EYFP and the I-A^k target antigen. Splenocytes from these F1 mice were injected into $MDR^{-/-}$ recipients with or without I-A^k-gelonin, and one day later the spleens were harvested from these mice for flow cytometry. We have shown previously that a high dose (2 mg/kg) depletes wild type donor cells (20) therefore we titrated down to identify a dose that would deplete $MDR^{-/-}$ cells that had acquired target antigen, but not the original donor cell population. This experiment was repeated several times with

different doses of I-A^k-gelonin. A representative experiment is shown in fig 4, with example dot plots (fig 4a). After injection of splenocytes alone, MHC transfer was seen with recipient ($EYFP^-$) cells expressing donor I-E^k (0.56±0.12% of total splenocytes). Injection of Anti I-A^k-gelonin reduced this population of cells (fig 4b) without affecting the I-E^{k+} donor ($EYFP^+$) population (fig 4c). Only at doses of 1mg/kg or higher were I-E^{k+} donor cells affected (fig 4c). Depletion of the target population showed a dose dependent response, with an almost 50% decrease in the target population at a dose of 0.5mg/kg I-A^k-gelonin.

Having demonstrated the principle of ultra selective killing using this method by showing that only recipient but not donor cells expressing the target molecules are killed, we went on to optimise this technique in an organ transplant model because of its real life relevance. After organ transplantation, donor antigen presenting cells traffic to the spleens of recipients (21) where intercellular MHC transfer can occur (22).

3.3 *In vivo* application ofAnti I-A^k-gelonin immunotoxin – organ transplantation

EYFPBL/6 x CBA F1 mice were not suitable for use as organ donors for titration studies as staining for EYFP in spleen sections using immunohistochemistry resulted in very high background, unsuitable for the accurate assessment of relatively rare events (data not shown). Flow cytometry also proved to be inappropriate as the number of donor cells in recipient spleens following organ transplantation is much lower than that following splenocyte injection.

Therefore, BL/6 x CBA F1 mice were given BrdU in their drinking water to label dividing cells, in order to identify donor cells that have trafficked to recipient spleens by immunohistochemistry. Staining of hearts and kidneys of these mice confirmed that up to 90% of MHC class II^+ cells also stained positive for BrdU (representative pictures of a kidney section are shown in fig 2b-d), with 80% of $CD11c^+$ dendritic cells also $BrdU^+$. BrdU labelling of MHC class II^+ cells was investigated in the kidney and heart of mice fed BrdU in drinking water for between 10 and 28 days, and 21 days was chosen as the optimal time to give maximum labelling (data not shown). As BrdU intercalates into DNA, histological staining highlights the nucleus of labelled cells, clearly distinguishable from cell surface staining for MHC molecules when we detect cells that have acquired donor MHC molecules.

Hearts and kidneys from BL/6 x CBA F1 mice treated with BrdU were transplanted into $MDR^{-/-}$, or wild type FVB, recipients with or without I-A^k-gelonin. One day after transplant the spleens were harvested for immunohistochemistry. $BrdU^+$ cells could be readily detected (fig 2e-g). After heart transplantation into $MDR^{-/-}$ mice, recipient cells that had taken up donor MHC class II, the target population (I-$A^{b+}BrdU^-$ cells) were 81±33% of the total $BrdU^+$ population. After injection with 0.25, 0.375 or 0.5mg/kg I-A^k-gelonin, this percentage fell to 62±24%, 24±5.7% and 15±4.5% respectively, although this reduction did not reach statistical significance. The non-target population was spared - the percentage of donor cells expressing donor MHC class II (I-$A^{b+}BrdU^+$ cells) did not decrease after Anti I-A^k-gelonin treatment, being 27±6.2% with no treatment, and 32±3% after the injection of 0.5mg/kg Anti I-A^k-gelonin (fig 5a).

After kidney transplantation into $MDR^{-/-}$ mice, recipient cells that had taken up donor MHC class II (I-$A^{b+}BrdU^-$ cells, the target population) were 21.41±3.49% of the total $BrdU^+$ population. After injection with 0.25, 0.375 or 0.5mg/kg I-A^k-gelonin, this percentage fell to 17±4.9%, 5.9±2.1% and 18±6% respectively. This reduction was significant at 0.375mg/kg Anti I-A^k-gelonin($p=0.0475$). Again, the non-target population was spared - the percentage of donor cells expressing donor MHC class II (I-$A^{b+}BrdU^+$ cells) did not decrease after Anti I-A^k-gelonintreatment, being 17±4.8% with no treatment, and 24±6.6% after injection of 0.5mg/kg Anti I-A^k-gelonin (fig 5b).

The ultra selective depletion was dependent on the target population being MDR deficient. When wild type FVB mice were used as recipients instead of $MDR^{-/-}$, Anti I-A^k-gelonin injection had no effect on either the I-$A^{b+}BrdU^-$ or I-$A^{b+}BrdU^+$ populations (fig 5c).

Although BrdU labelling of donor cells was efficient, it was not 100% effective. Some donor cells (toxin resistant) would persist and affect the observed efficiency of the depletion. An attempt to correct for this is shown in the discussion section.

4. Discussion and conclusion

Here, we describe a novel method of cell depletion that potentially can achieve a higher level of specificity than existing techniques. This may provide a route for other investigators to study various immunological phenomena in different models. The principle governing the success of this technique is that the target cell population is exquisitely susceptible to the toxic effect of the chosen toxin. By using an antibody to deliver the toxin to cells expressing the target molecule, only $MDR^{-/-}$, target molecule expressing cells are depleted. MDR sufficient cells, even if they express the target molecule, are spared. The mechanism of $MDR^{-/-}$ cells sensitivity to toxin is generally thought to be inability to remove the toxin from the intracellular compartment. In addition, lysosomal accumulation of toxin had also been implicated in the case of gelonin. Furthermore, unknown secondary changes in the cells subsequent to loss of P-gp being responsible for the sensitivity cannot be excluded. However, whatever the mechanism, the generalizability still holds, as the model describe here depends on target expressing cells deficient in P-gp being killed while wild type cells are spared. Therefore, regardless of which mechanism is responsible for the killing, the method described here is valid.

By depleting recipient cells that have acquired donor MHC class II molecules using immunotoxin technology and the $MDR^{-/-}$ mouse strain which is ultra-sensitive to certain drugs, we have shown that it is possible to study the consequences of transfer of these molecules without the need for complex models and adoptively transferred cells, thus allowing investigations to be carried out under normal conditions. A trend of depletion was observed, without non-specific killing.

It is possible that our immunotoxin favours the dissociation of the membranes/MHC molecules acquired through trogocytosis instead of killing the cells but we think this is unlikely as we have demonstrated the internalisation of the antibody which is required to deliver the toxin. It may also favour the dissociation of fused donor/recipient cells. However, as these would have been gated out in the scatter plot during flow cytometric analysis, this possibility is also not very likely. It can be argued that trogocytosis might render recipient cells more sensitive to the immunotoxin. However, we think that this is unlikely as figure 4c shows that the percentage of target expressing donor cells is not affected. MHC exchange is a bi-directional process as we have previously demonstrated (22), so these recipient cells would have undergone trogocytosis and taken up donor MHC molecules as well, but yet, they were not susceptible to killing.

Transfer of pgp has been reported in tumour cells. It is not known whether it is also transferred in lymphoid cells. Nonetheless, our technique has been shown to be effective. It is possible that not enough P-gp is acquired by target cells to make them resistant. Indeed, we have previously shown that high doses of the same immunotoxin as the one we used here can overcome Pgp expression and kill wild type cells expressing the target molecules (20).

Immunotoxin synthesis is a relatively simple procedure and does not require any specialist equipment. Furthermore, clinical grade immunotoxins are already in use in humans. As a result, a wide range of toxins are available. We have chosen the plant based ribosomal inactivating protein gelonin because it cannot enter cells on its own without being delivered to target cells by an antibody. Upon internalisation of the immunotoxin, the acidic pH within the endosomal compartments results in the reduction of the disulfide bond which is formed between the $F(ab')_2$ fragments and gelonin during the conjugation process. The dissociated gelonin can then exert its toxic effect. Any free gelonin released from the dead target cells would not be toxic as it cannot cross the cell membrane.

Having demonstrated the principle of selective depletion of $MDR^{-/-}$ cells in a splenocyte injection model, we proceeded to optimise the selectivity and effectiveness in a vascularised organ transplantation model. After BrdU administration, not all donor cells positive for donor MHC class II became labelled with BrdU, suggesting that a small population of cells is resistant to BrdU labelling. The reason for this is not immediately apparent, and is not the main focus of this work.

As not all MHC class II positive cells in donor kidneys and hearts were labelled with BrdU, these cells when trafficked to the spleens of recipients after transplantation would have been falsely identified as recipient cells. They would also express donor MHC class II molecules, and masquerade as our target population. Since they are donor derived, they would be resistant to Anti $I\text{-}A^k$-gelonin killing, making the immunotoxin appear less effective. We have performed calculations to estimate the size of this false negative population. 24% of donor MHC class II positive cells in the heart failed to be labelled by BrdU and would therefore masquerade as target cells, and so this percentage was used to calculate the actual number of false negative cells in the spleens of

recipients to give an estimate of the degree of true depletion. After subtraction of this estimated false negative population, the corrected size of the target I-$A^{b+}$$BrdU^{-}$ population was 74+34% of the total $BrdU^{+}$ population. After injection with 0.25, 0.375 or 0.5mg/kg I-A^{k}-gelonin, this percentage fell to 53+24%, 13+6.4% and 5.5+3.7% respectively, although again this reduction did not reach statistical significance (Fig 5d). 15% of donor MHC class II positive cells in the kidney failed to be labelled by BrdU, and therefore this percentage was used to estimate the false negative population in the spleens of kidney transplant recipients. After subtraction of this estimated false negative population, the corrected size of the target I-$A^{b+}$$BrdU^{-}$ population was 18.2+2.7% of the total $BrdU^{+}$ population. After injection with 0.25, 0.375 or 0.5mg/kg I-A^{k}-gelonin, this percentage fell to 12.6+4.8%, 1.1+1.1% and 13+5.3% respectively. This reduction was again significant at 0.375mg/kg I-A^{k}-gelonin ($p=0.0175$, fig 5e). This population was represented as grey bars in a stacked graph, while the corrected level of depletion was plotted as black bars below (fig 5d-f). Fig 5f shows that after the same correction for false negativity has been applied, the toxin still did not affect the target population in control FVB recipients.

In kidney transplant recipients, the highest dose (0.5mg/kg) was not as effective as the next highest dose used (0.375mg/kg). The reason for this is not clear.The most effective dose was different for heart and kidney transplants, at 0.5 and 0.375mg/kg respectively. There are higher numbers of donor passenger leukocytes present within kidneys than hearts, and it may be that at the higher dose this population was being targeted, rather than the recipient population which we intended to deplete. Due to these reasons, the dose of immunotoxin should be titrated for different applications.

The principle of exploiting increased susceptibility to immunotoxin killing as a novel method to deplete target populations of cells can also be extended for different purposes. For example, although depletion of cells that express a certain surface marker can be done efficiently using depleting monoclonal antibodies, this method would not allow the specific depletion of only a subset of target expressing cells. For example, CD44 is an activation/memory cell surface marker for both $CD4^{+}$ and $CD8^{+}$ T cells. Selective depletion of the memory $CD4^{+}$ T cell ($CD4^{+}CD44^{+}$) subpopulation would not be possible using depleting CD44 monoclonal antibodies as memory $CD8^{+}$ T cells would also be killed. If a genetically modified mouse was created in which only the $CD4^{+}$ T cells were $MDR^{-/-}$, using for example the cre/lox system of genetically modified mice that are already available to knock out genes of interest in specific cell populations, then using the principle demonstrated here,

it would be possible to selectively deplete memory $CD4^+$ T cells only for *in vivo* immunological studies, although we acknowledge that dedicated lines of genetically modified mice have to be bred from existing cre/lox mice under different promoters to achieve specific purposes, which may be time consuming.

We have shown here that depletion with one dose of immunotoxin can be effective in our model. The exact dosing schedule would also need to be titrated for individual models. For example, the model that we have chosen here (MHC transfer after organ transplantation) is a continual process, and if long term depletion is required, repeated dosing may be necessary.

We have already successfully demonstrated the principle of selective killing here by using $MDR^{-/-}$ cells which had increased susceptibility to killing: after kidney transplantation, the depletion was highly effective, but less so after heart transplantation. Therefore, the data only reach statistical significance under very specific conditions and not in all the conditions tested. Further titration using different doses and timing may increase the effectiveness of this approach. In addition, this may be due to the fact that the immunotoxin has a relatively narrow therapeutic window. This can, in theory can be made even more robust by using animals that overexpress the MDR genes, which will confer an even higher degree of resistance to killing, as the source of cells that need to be spared. For example, using the model of transplantation here, animals that overexpress the MDR gene (such animals already exist (23)) would be used as donors, while the recipient animals are $MDR^{-/-}$.

Trogocytosis is a complex phenomenon. It is a continual process that takes place through the life of the donor graft (22). Therefore, the residual population of target cells that have not been depleted may have just acquired the donor MHC molecule and have not had time to be killed by the toxin.

One obvious application of this novel technique is to deplete recipient cells that have acquired donor MHC molecules after organ transplantation to elucidate the effect of MHC transfer on graft survival and therefore its contribution to the alloimmune process. This pathway of antigen presentation had been highlighted as the main pathway for allograft rejection (24, 25). The technique described here is well suited for the further study for this.

Immunotoxins target cells with the appropriate cell surface molecules. It is possible that after trogocytosis, some of the exchanged molecules are internalised and therefore, not available for immunotoxin binding. However, it is unlikely that all the exchanged molecules are internalised leaving none on the cell surface. Even if this is the case, these cells with all the acquired molecules internalised are likely to be functionally distinct to cells that are still expressing the acquired molecules on their surfaces, e.g. in their ability to present the acquired molecules as antigens.

Depletion of specific populations of cells *in vivo* is an extremely powerful technique to study the function of the population concerned. Here, we describe a novel method that can potentially add further specificity to this strategy. The technique used is relatively simple and can be adopted by most investigators needing highly targeted depletion of specific cell populations.

Author Contributions

K. Brown and W Wong designed and conducted the experiments and wrote the paper. L Meader, A Nowocin and LA Edwards conducted the experiments. LH Cheung and M Rosenblum designed the experiments and synthesised the gelonin. R Smith designed the experiments and wrote the paper.

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Conflict of interest

The authors declare that they have no competing interest.

Ethical Standard

All animal work was approved by the King's College London ethical committee.

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Legend to figures

Figure 1. Schematic representation of the antibody-gelonin immunotoxin $MDR^{-/-}$ principle. The immunotoxin will bind to I-A^k positive donor cells A, but not recipient cells that do not normally express this molecule on their cell surface (not illustrated in this diagram), unless they have acquired it from the donor B. Donor cells are MDR sufficient, enabling them to pump out the toxin gelonin which is non toxic extracellulary (top panel) C. MDR deficient cells may also be sensitive to toxin killing due to lysosomal accumulation of the immunotoxin complex D. Recipient cells that have acquired the donor MHC class II molecule, however, are MDR deficient and thus, unable to export the gelonin and are killed (bottom panel) E.

Figure 2. Immunohistochemistry. a Internalisation of anti-I-A^k antibody by CBA cells which express the I-A^k antigen. Confocal microscopy shows the same cell, highlighted by the red arrows, from 3 different angles, confirming that the fluorescent staining seen within the cell is within the cytoplasm of the cell. The bottom panel shows a transverse section of the cell taken at the point of the horizontal red line, with the right panel showing a section of the cell taken at the point of the vertical red line.

Staining of a kidney section from a BL/6 x CBA F1 mouse treated with BrdU in drinking water with anti-BrdU (b) and anti-I-A^b (c) antibodies (composite in d) shows the majority of I-A^{b+} cells are also $BrdU^+$ (yellow arrows), however a minority of I-A^{b+} cells are $BrdU^-$ (white arrows). e, g, h. Staining of a spleen section from an $MDR^{-/-}$ recipient of a BrdU-treated BL/6 x CBA F1 transplant with anti-BrdU (e) and anti-I-A^b (f) antibodies (composite in g) shows that $BrdU^+$I-A^{b+} cells can be detected in the recipient spleen after transplant. BrdU was stained with PE and visualised by fluorescent microscopy, while I-A^b was stained with the horseradish peroxidase substrate NovaRED and visualised by light microscopy. For display purposes I-A^b stained images have been altered so that positive staining is shown in green to allow for overlay with BrdU staining. All x200 magnification.

Figure 3. a. Binding of antibody and $F(ab')_2$ fragments to CBA target cells. Cells were incubated with antibody or $F(ab')_2$ fragments, before staining with a fluorochrome-labelled antibody of the same, or an irrelevant, clone. Pre-incubation with antibody or $F(ab')_2$ fragments inhibited binding of the labelled

antibody of the same clone to a similar degree, while binding of the irrelevant clone was unaffected (upper panel). Example of FACS analysis (lower panel). b. SDS PAGE gel showing antibody (150 kDa), $F(ab')_2$ fragments (110 kDa), and I-A^k-gelonin conjugate (140 kDa). A small band of unconjugated $F(ab')_2$ fragments remains in the I-A^k-gelonin preparation, even after purification. c. Binding of antibody and I-A^k-gelonin to CBA target cells. Cells were incubated with antibody or I-A^k-gelonin, before staining with a fluorochrome-labelled antibody of the same, or an irrelevant, clone. Pre-incubation with antibody or I-A^k-gelonin inhibited binding of the labelled antibody of the same clone to a similar degree, while binding of the irrelevant clone was unaffected (upper panel). Example of FACS analysis (lower panel).

Figure 4. Splenocytes from BL/6 EYFP x CBA F1 mice were injected into $MDR^{-/-}$ recipients, with increasing doses of I-A^k-gelonin. Spleens were harvested 20 hours later for flow cytometry. a. Example dot plots following treatment with (right-hand panel) or without (left-hand panel) I-A^k-gelonin. b. The percentage of I-$E^{k+}EYFP^-$ cells (recipient cells that have acquired donor MHC class II molecules - the target cell population) decreased with increasing doses of I-A^k-gelonin. c. The percentage of I-$E^{k+}EYFP^+$ cells (donor cells, which should be resistant to killing) did not decrease, except at the highest dose of 1mg/kg. n = 3 in each group.

Figure 5 BrdU-treated BL/6 x CBA F1 mice were used as organ donors and recipient spleens were harvested 1 day later for immunohistochemistry. The percentage of I-$A^{b+}BrdU^-$ cells (recipient cells that had acquired donor MHC class II molecules, and the target cell population) decreased with increasing doses of I-A^k-gelonin, following heart (a) and kidney (b) transplantation into $MDR^{-/-}$ recipients. The percentage of I-$A^{b+}BrdU^+$ cells (donor cells, which should be resistant to killing) did not decrease. The percentage of I-$A^{b+}BrdU^-$ cells did not decrease when wild type FVB mice were used as recipients (c). n = 4 in each group.

Figure 5 def.Correction for less than 100% efficiency of BrdU labelling. The estimated false negative population was plotted in stacked graphs for heart (d) and kidney (e) transplantation into $MDR^{-/-}$ recipients; into FVB recipients (f). n = 4 in each group.

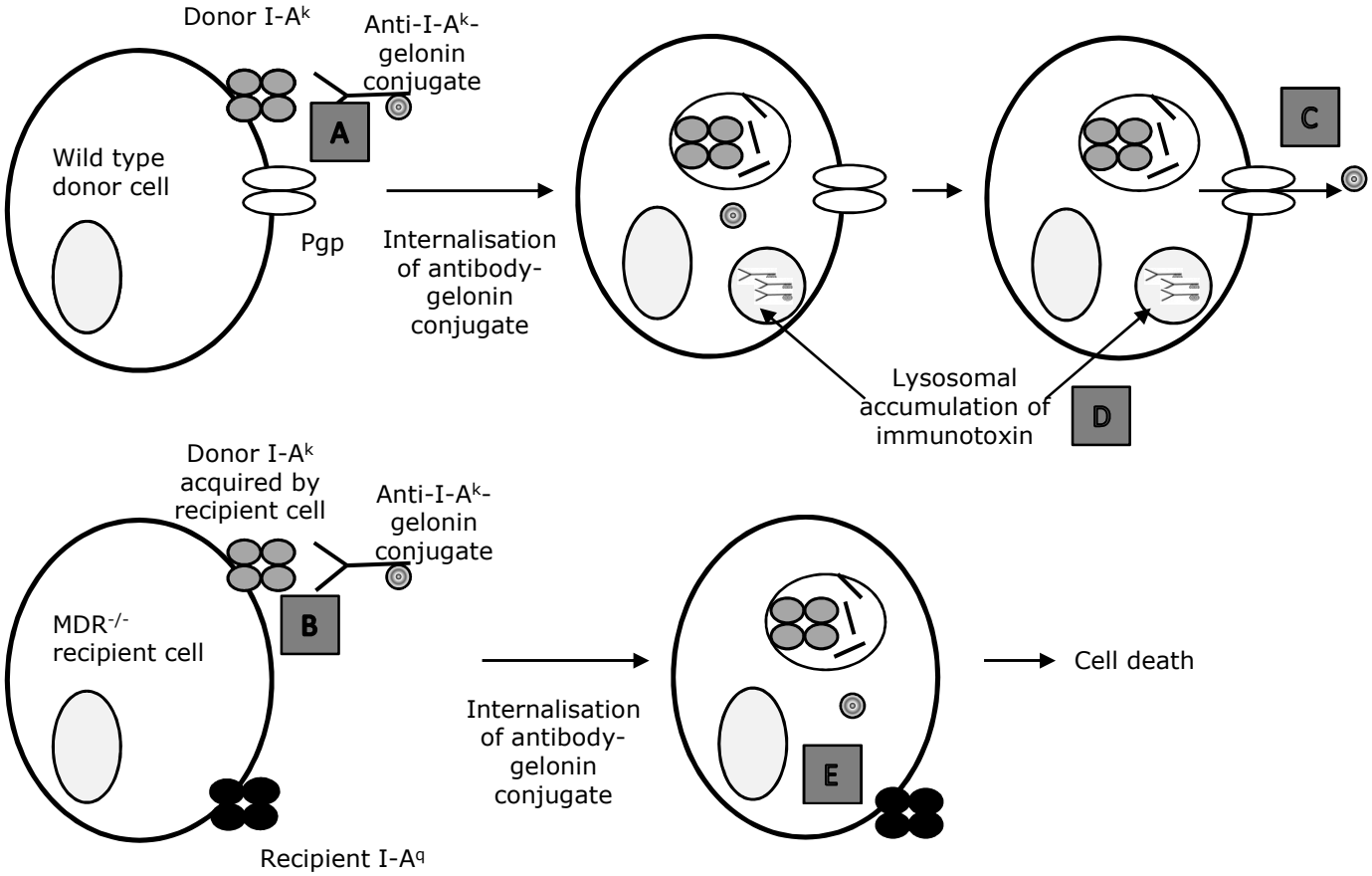
Donor I-A^k
Anti-I-A^k-gelonin conjugate
A
Wild type donor cell
Pgp
Internalisation of antibody-gelonin conjugate
C
Lysosomal accumulation of immunotoxin
D
Donor I-A^k acquired by recipient cell
Anti-I-A^k-gelonin conjugate
B
MDR$^{-/-}$ recipient cell
Internalisation of antibody-gelonin conjugate
E
Cell death
Recipient I-A^q

Figure 1